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Transfer of microbiota from hatchery environment to newly hatched chicks

Daniela Karasova[a], Vladimir Babak[a], Marcela Faldynova[a], Alena Sebkova[a], Hana Prikrylova[a], Jiri Volf[a], Zdenek Tvrdon[b], Marian Foltyn[b], Jana Rajova[a], Ivan Rychlik[a,*]

[a] Veterinary Research Institute, Hudcova 70, Brno, 62100, Czech Republic
[b] Vykrm Trebic, Karlov 196, Kutna Hora, 28401, Czech Republic

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ABSTRACT

In this study, the microbiota from dead chick embryos and eggshell waste from hatcheries was compared with the caecal microbiota of one- and four-day-old chicks to identify taxa characteristic of each sample type as well as taxa that can be transferred from hatcheries to chicks and farms. Altogether 260 samples were analysed by 16S rRNA sequencing. Major microbiota members of each sample type were not found in other sample types and the carry-over of bacteria from one sample type to another was uncommon. The caeca of one-day-old chicks in hatcheries were colonised by *Clostridium disporicum, C. paraputrificum, C. tertium, Terrisporobacter* and *Zhenhengia* (*Epulopiscium*). The presence of these taxa seem to be dependent on feed intake since once chicks started with feed intake after placement at farms, these bacteria disappeared from the caecal microbiota. *Escherichia coli* and *Enterococcus faecalis* were found in hatchery samples and also in one-day-old chicks suggesting that these species originate from the hatchery environment. However, both *E. coli* and *Ent. faecalis* were found only in 10-20 % of chicks leaving the hatcheries.

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Introduction

Chickens represent one of the most important sources of animal protein for the human population. To cover the demands of the human food chain, poultry production is highly optimised, starting from egg production in reproductive flocks, through to egg incubation and hatching of chicks in hatcheries up to the transport of chicks to farms. One of the most critical parts of the whole production is the first week after the placement of chicks on farms when increased mortality is commonly recorded (Yassin et al., 2009; Yerpes et al., 2020). Since this mortality can be controlled by antibiotics, the major causes of the losses are likely of bacterial origin.

Colonisation of chicks from hatcheries represents a specific case, different from the colonisation of the chicks that would be hatched in nests (Glendinning et al., 2022; Marcolla et al., 2023; Faldynova et al., 2024; Rychlik, 2026). There are multiple reports describing the development of gut microbiota in chicks from hatcheries, but these commonly compared the microbiota composition in 1-, 2- or 3-week-old and older chickens (Stanley et al., 2013; Videnska et al., 2014; Den Hartog et al., 2016; Gao et al., 2017; Xi et al., 2019). However, peaks in mortality are recorded around day 3 or 4 of life and it is not unusual that losses start immediately on the day the chicken is placed on the farm (Yassin et al., 2009; Yerpes et al., 2020). Studies on the microbiota composition of 1- or 2-week-old chickens therefore characterise the microbiota already after the most critical part of life. Rather surprisingly, there are only a few reports on the gut microbiota of chicks when leaving hatcheries (Videnska et al., 2014; Projahn et al., 2017; Glendinning et al., 2019; Huang et al., 2023; Manders et al., 2025; Vieira et al., 2025). Moreover, we are not aware of any report analysing microbiota in the hatchery environment. Around 20 % of eggs are lost during embryonic development. Irrespective of the reasons for embryonic death, such eggs are subjected to infections and bacterial multiplication. These bacteria then contaminate the hatchery environment but whether these are the same as those in the eggshell waste or the caecal microbiota of chicks leaving hatcheries is not known. In addition, the accumulation of eggshell waste during hatching, with remains of biological material from the eggs present in an environment with 37°C and 80 % humidity, represents ideal conditions for the multiplication of bacteria to which hatching chicks are exposed from the very first moments of their life.

Despite the importance of one-day-old chick quality for the whole production system, there are no studies comparing microbiota composition in hatcheries and in newly hatched chicks without any bias,

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* Corresponding author.
E-mail address: rychlik@vri.cz (I. Rychlik).

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i.e. by DNA sequencing. We partially addressed this topic when we analysed eggshell microbiota of eggs with correctly developing embryos. These samples were dominated by microbiota characteristic for the caecum of adult hens and litter microbiota (Cressman et al., 2010; Johnson et al., 2018; Volf et al., 2021; Kubasova et al., 2022). In a limited number of samples, we also analysed the microbiota from eggshell waste in hatcheries (Rychlik et al., 2023). This showed that the most common genera included *Brevibacterium* (Actinobacteria); *Chryseobacterium* and *Flavobacterium* (Bacteroidetes); *Enterococcus, Clostridium paraputrificum* and *Clostridium disporicum* (Firmicutes); and *Acinetobacter, Comamonas, Enterobacter, Escherichia, Proteus* and *Pseudomonas* (all Gammaproteobacteria). *E. coli* and *Enterococcus* spp. are commonly reported in chicks after placement on farms, indicating the potential transfer of these taxa from hatcheries to farms (Projahn et al., 2017; Glendinning et al., 2019; Huang et al., 2023) and spore-forming representatives from the families Lachnospiraceae, Oscillospiraceae or Ruminococcaceae begin to dominate caecal microbiota of chicks around 1 week of age (Stanley et al., 2013; Videnska et al., 2014; Xi et al., 2019). Which of these taxa are found in hatcheries and which are likely acquired from the farm environment after chick placement on farms is not known.

In this study, we therefore analysed and compared microbiota from dead embryos, microbiota in eggshell waste from the floors of hatcheries, and caecal microbiota of one- and four-day-old chicks. The initial aim of this study was to identify taxa characteristic for each sample type. However, the real main aim was to identify bacteria that were present in similar abundance in samples from the hatchery environment and in caecal microbiota of chicks, since those are taxa that can be transferred from hatcheries to chicks and farms.

Material and methods

Ethical statement

The handling of animals in the study was performed in accordance with current Czech legislation (Animal Protection and Welfare Act No. 246/1992 Coll. of the Government of the Czech Republic). The specific experiments were approved by the Ethics Committee of the Veterinary Research Institute followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permit number MZe2405 approved on March 5, 2023).

Collected samples

Microbiota composition was determined in 260 samples, of which 15 samples originated from dead embryos originating from 5 different batches of eggs, 129 samples from eggshell waste collected in a single broiler chick producing hatchery over a period of two years with one collected sample per one batch of eggs, 49 samples from the caeca of one-day-old chicks originating from 16 different chicken flocks and 67 caecal content samples were collected from four-day-old chicks originating from 24 different flocks. One-day-old chicks were defined as chicks without any access to feed and any physical contact with the farm environment, i.e. these were taken either from hatcheries or after transport to farms but before offloading chicks from the truck cargo space to the barns. Whether the one-day-old chicks were collected at farms or at the hatchery, transport time to the Veterinary Research Institute was always between 1 - 2 hours. Day 4 was selected since peak in mortalities can be commonly recorded at farms at this age. Finally, samples from 1-day-old and 4-day-old chicks were collected independently, i.e. not from the same flocks. Non-chicken samples were immediately stored at -20°C until DNA purification. All chicks were subjected to CO_2 anesthesia followed by decapitation. Caecal contents were collected and stored at -20°C until DNA purification.

In an additional 98 one-day-old chicks, *E. coli* presence was determined by cross-streaking caecal contents on MacConkey agar plates followed by aerobic incubation at 37°C for 24 hours. Red colonies characteristic for *E. coli* were visually identified. Whenever appropriate, colonies with less characteristic morphology were tested by MALDI Biotyper. Clostridia were grown on Brain Heart Infusion (BHI), Wilkins-Chalgren Agar (WCHA) and blood agar both under aerobic and anaerobic conditions at 37°C for 48 hours. Growing colonies were selected, subcultured and subjected to MALDI Biotyper identification.

Purification of total DNA from caecal digesta

Each collected sample was homogenised using a MagnaLyser (Roche) and the homogenate was subjected to DNA purification using QIAmp DNA Stool kit (Qiagen). Purified DNA was stored at -20°C.

Microbiota characterisation by sequencing over V3V4 variable region of the 16S rRNA gene

The 16S rRNA sequencing was performed following the protocol described previously (Kubasova et al., 2022). The raw sequence reads have been deposited in the NCBI Short Read Archive under accession number PRJNA1522852.

Data evaluation and statistics

Initial analysis of microbiota composition was performed with QIIME 2 v2021.2 (Bolyen et al., 2019). Raw sequence data were demultiplexed, quality filtered, and sequencing primers were clipped using Je (Girardot et al., 2016) and fastp (Chen et al., 2018). Resulting sequences were denoised with DADA2 (Callahan et al., 2016). Taxonomy was assigned to amplicon sequence variants (ASV) using the q2-feature-classifier (Bokulich et al., 2018) and classify-sklearn naïve Bayes taxonomy classifier against the Silva 138 database (Quast et al., 2013). All the software tools were used with default settings. A partial 16S rRNA sequence was used and exported as the key identifier of each ASV (Supplementary File 1). If appropriate, this sequence was used for manual BLAST search against GenBank database for up-to-date taxon identification. Identified species names are used in this paper for clarity reasons, however, one has to be aware that partial sequence of 16S rRNA gene is only a proxy for bacterial species identification and each species name used in this report should be understood as describing species related isolates. Principle Coordinate Analysis (PCoA) graphs were generated by Qiime using Bray-Curtis metrics. Column figures were produced from the ASV table in Microsoft Excel. All the remaining analyses, statistics and figures were produced in R (version 4.5.2).

PCoA clustering and randomized residual permutation procedure (RRPP)

Randomized Residual Permutation Procedure (RRPP library, function rrpp with 10,000 permutations) was used to determine global differences among all the groups. Differences in position (average distances of centroids in ordination space) and in dispersion (average distances of individual data points from centroids) with P values lower than 0.05 were considered as significant.

Linear discriminant analysis effect size (LEfSe) analysis

Data for LEfSe analysis were prepared using phyloseq library while LEfSe itself was carried out with microbiomeMarker library. The first step included selection of significantly different taxa using Kruskal-Wallis test and P-values were then adjusted for false discovery rate (FDR) using Benjamini-Hochberg correction. Only taxa with FDR-adjusted P-values <0.05 were used to estimate the effect of the size (linear discriminant analysis (LDA) score) using the LEfSe algorithm. The threshold value for LDA (lda_cutoff) was set to a value 3.0 to identify only robust biomarker taxa characteristic for each sample type.

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Correlation analysis and hierarchical clustering

Correlation analysis was produced using Spearman correlation coefficients for 30 of the most common families. Mutual correlations were then visualised using a heat map produced from correlation coefficients. The same families were analysed also by abundance-based hierarchical clustering. Bray-Curtis dissimilarity index was used as a metric and Ward's linkage together with k-clustering were used for the clustering itself.

Abundance distribution using kernel density estimation (KDE)

Probability Density Function (PDF) was determined using Kernel Density Estimation.

Significance of differences in PDFs of particular taxa in different sample types was determined by the Wasserstein distance test.

Results

Sequence data used in downstream analyses

In total, 16,659,051 reads were obtained for 260 analysed samples with an average coverage of 64,073 reads per sample. Minimal and maximal sample read coverages were 2,075 and 423,771 reads, respectively. There were 23 samples with coverage lower than 10,000 reads, 24 samples with 10,001 to 20,000 read coverage, 91 samples with 20,001 to 40,000 read coverage, 36 samples with 40,001 to 60,000 read coverage, and 86 samples with coverage higher than 60,000 reads. This means that we should have detected bacteria forming 0.01% of the total population in most of the samples but could have missed those that had lower representation. Whenever we report on the absence of a particular bacterium, this should be understood this bacterium is indeed absent or present but below the detection limit.

Gross comparison of tested samples

PCoA clustering based on microbiota composition separated samples from dead embryos, eggshells, and one- and four-day-old chicks into different clusters (Fig. 1A, Randomized Residual Permutation Procedure, $P < 0.05$). Taxa responsible for this separation as well as shared taxa were identified in subsequent analyses.

Altogether 4,795 ASVs were recorded in at least one sample, and these were assigned to 42 different families. The 30 major families that formed more than 90 % of the total microbiota in each sample type were initially analysed. Brevibacteriaceae, Dysgonomonadaceae or Aerococcaceae were found only in dead embryo samples. Weeksellaceae, Moraxellaceae or Oxalobacteraceae were characteristic for eggshell waste, whereas Lactobacillaceae, Butyricicoccaceae, Oscillospiraceae or Ruminococcaceae were found exclusively in the caeca of 4-day-old chicks (Fig. 1B). The caecal microbiota of 1-day-old chicks did not exhibit any specific family since representatives of Peptostreptococcales-Tissierellales, Lachnospiraceae, Enterococcaceae, Enterobacteriaceae and Clostridiaceae common to this sample type were found also in other sample types (Fig. 1B).

Biomarker families for individual sample types

Using LEfSe analysis, families characteristic for each sample type were identified. Eggs with dead embryos were infected by Comamonadaceae (represented mainly by the genus *Comamonas*), Morganellaceae (genus *Proteus*), Peptostreptococcales-Tissierellales (*Helcococcus*) or Brevibacteriaceae (*Brevibacterium*) (Fig. 1C). Eggshell waste was dominated by Moraxellaceae (genus *Acinetobacter*), Weeksellaceae (*Chryseobacterium*), Pseudomonadaceae, Oxalobacteraceae (*Janthinobacterium*), Xanthomonadaceae (*Stenotrophomonas*), Sphingobacteriaceae or Flavobacteriaceae. The caeca of one-day-old chicks were colonised by representatives of Clostridiaceae (*Clostridium tertium, C. paraputrificum* and *C. disporicum*) and Peptostreptococcaceae (genus *Terrisporobacter*) (Fig. 1C). The caeca of four-day-old chicks were colonised by Lachnospiraceae, Enterobacteriaceae, Lactobacillaceae, Oscillospiraceae, Ruminococcaceae, Butyricicoccaceae, Erysipelatoclostridiaceae or Streptococcaceae (Fig. 1C). Families which were common in more than one sample type and therefore could not be assigned by LEfSe as a biomarker of a particular sample type included Enterococcaceae, Bacteroidaceae, Dysgonomonadaceae, Aerococcaceae or Vagococcaceae (Fig. 1C).

Co-existence of particular families in samples from dead embryo, eggshell waste and chicks

Correlation analysis at the family level resulted in four major clusters. The first cluster was formed by families Pseudomonadaceae, Moraxellaceae, Xanthomonadaceae, Weeksellaceae, Comamonadaceae, Planococcaceae, Sphingobacteriaceae, Flavobacteriaceae and Aeromonadaceae, i.e. families common to eggshell waste (Fig. 1D). The second clearly separated cluster was formed by families Morganellaceae, Enterococcaceae, Enterobacteriaceae and Bacteroidaceae. Of these, Enterococcaceae and Bacteroidaceae could not be assigned to a particular sample type by LEfSe. Morganellaceae and Enterobacteriaceae, despite dominating in dead embryos and caecal microbiota of 4-day-old chicks by LEfSe, respectively, were found in increased abundance also in other sample types. The next cluster comprised families Clostridiaceae and Peptostreptococcaceae that were common in the caecal microbiota of one-day-old chicks, together with Brevibacteriaceae, Peptostreptococcales-Tissierellales, Aerococcaceae, Dysgonomonadaceae, Oxalobacteraceae, Selenomonadaceae and Staphylococcaceae that were common in the microbiota of dead embryos. The last cluster was formed by families Streptococcaceae, Lactobacillaceae, unclassified Bacilli, Oscillospiraceae, Ruminococcaceae, Erysipelatoclostridiaceae, Butyricicoccaceae and Lachnospiraceae, i.e. families common in the caeca of 4-day-old chicks (Fig. 1D).

There was a strong negative correlation between eggshell waste microbiota and caecal microbiota of 4-day-old chicks indicating the low likelihood of major families colonising the caeca of 4-day-old chicks originating from hatcheries. There was also a minor negative correlation between families characteristic of eggshell waste microbiota and families Morganellaceae, Enterococcaceae, Enterobacteriaceae and Bacteroidaceae. On the other hand, the latter four families moderately positively correlated with families characteristic for caecal microbiota of 4-day-old chicks.

Hierarchical clustering of families and genera from dead embryos, eggshell waste and chicks

Since LEfSe and correlation analysis provided partially conflicting results, another tool, hierarchical clustering based on abundance of 30 the most abundant families using Bray-Curtis dissimilarity index, was used. This analysis resulted in the formation of 4 different clusters (Fig. 2A). The first cluster was formed by bacterial families dominating in the caeca of 4-day-old chicks. Except for Bacteroidaceae and Streptococcaceae, the remaining families from this cluster were absent in other sample types. The next cluster comprised families moderately abundant in eggshell waste samples and an additional cluster was formed by families found in the samples from dead embryos. Finally, there was a cluster which included families common and highly abundant in eggshell waste, caeca of one-day-old chicks and ubiquitously distributed families Enterobacteriaceae and Enterococcaceae (Fig. 2A).

Abundance based clustering was performed also at the genus level (Fig. 2B). *Bacteroides, Lactobacillus*, uncl. Lachnospiraceae, *Flavonifractor*, uncl. Ruminococcaceae, uncl. Oscillospiraceae, *Butyricicoccus* and *Ruminococcus torques* group, i.e. bacteria usually dominating in caecal microbiota of 4-day-old chicks, formed the first cluster. *Klebsiella, Elizabethkingia, Sphingobacterium, Empedobacter, Staphylococcus, Enterobacter* and *Stenotrophomonas* formed the second cluster. These genera were

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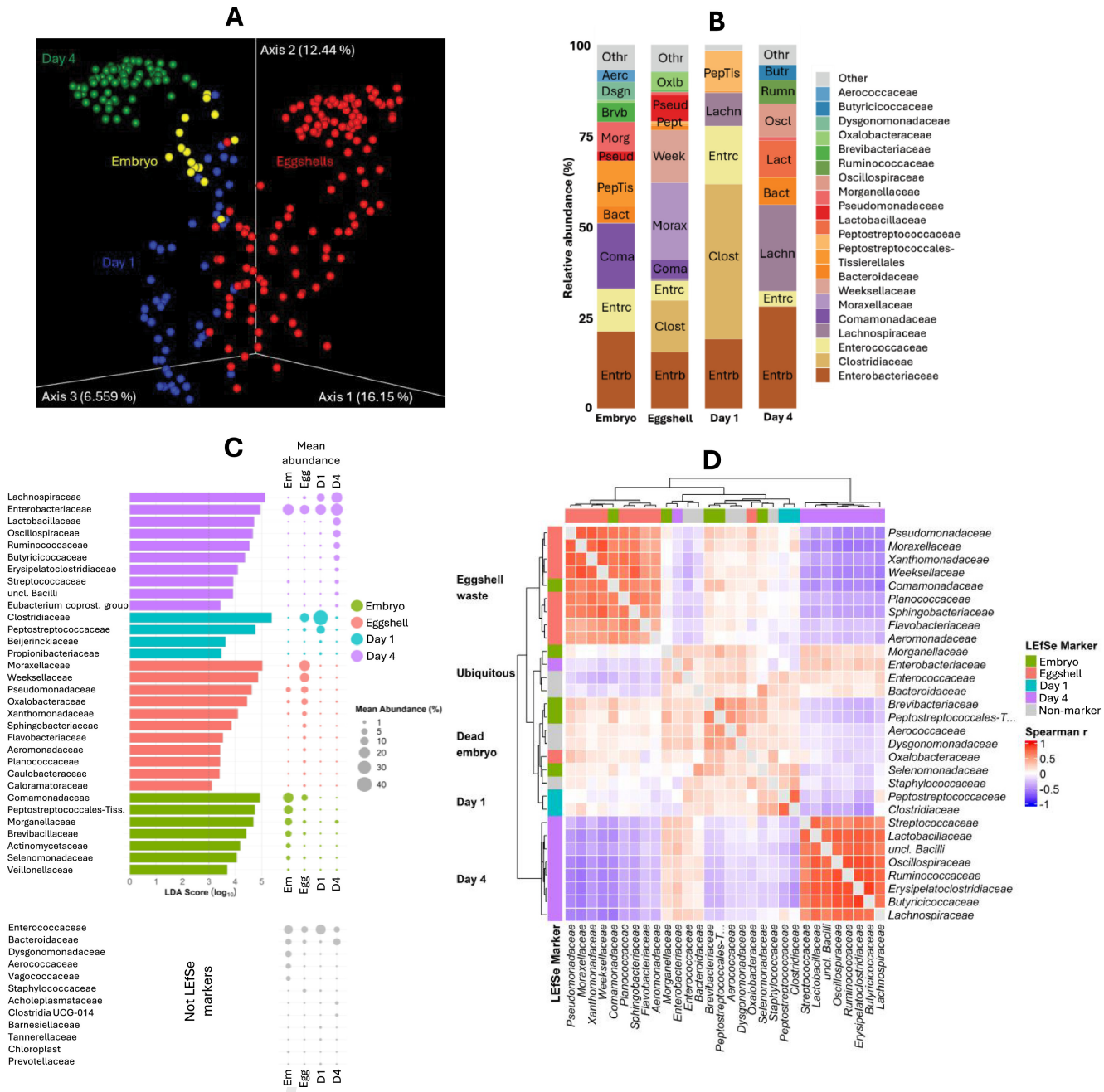


Fig. 1. Major differences in microbiota in eggs with dead embryos, eggshell waste and the caeca of one- and four-day-old chicks. Panel A, PCoA clustering of all samples analysed in this study. Panel B, major families recorded in each sample type. Panel C, LEfSe determined biomarker families for individual sample types, including families not specific to any sample type (in grey, "Not LEfSe makers"). Panel D, co-existence of particular families in samples from dead embryos, eggshell waste and chicken caecum. Colours of LEfSe marker are the same as in panel C.

characterised by a moderate abundance in eggshell waste samples. The third cluster was formed by bacteria dominating in the caeca of 1-day-old chicks, such as *Epulopiscium* (*Zhenhengia* after manual GenBank BLAST search using partial 16S rRNA sequence), *Terrisporobacter* and *Clostridium sensu stricto 1*, and ubiquitously distributed genera *Enterococcus* and *Escherichia*. The last cluster was formed by bacterial genera characteristic of dead embryos (*Brevibacterium, Proteiniphilum, Comamonas, Helcococcus* and *Proteus*), and bacteria of high abundance in eggshell waste (*Acinetobacter, Chryseobacterium, Janthinobacterium, Pseudomonas* and uncl. Enterobacteriaceae).

Detailed analysis of "non-specific" taxa

Since we were interested in bacteria which can be transferred from hatcheries to chicks, using data in Fig. 2, Bacteroidaceae, Morganellaceae, Lachnospiraceae, Clostridiaceae, Enterobacteriaceae and Enterococcaceae were identified as families common in caecal microbiota of 1- or 4-day-old chicks as well as in dead embryo or eggshell waste. Partial 16S rRNA sequences of ASVs from these families were therefore inspected in detail.

For Enterobacteriaceae and Morganellaceae, ASVs differing in individual nucleotides and therefore exhibiting similarities higher than 99 % were recorded across all samples. *Proteus mirabilis* was the representative of family Morganellaceae that dominated in dead embryo microbiota

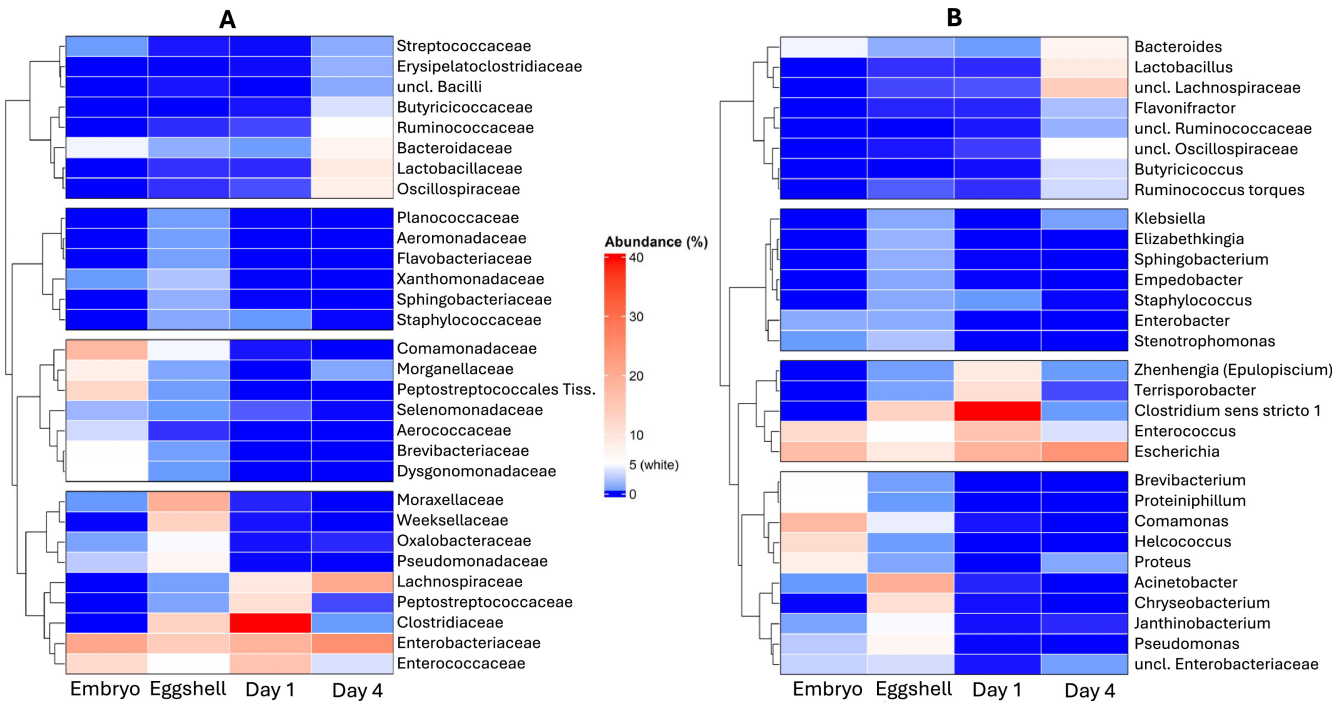


Fig. 2. Hierarchical clustering based on abundance of individual taxa in tested samples. Panel A, hierarchical clustering of 30 the most abundant families. Panel B, hierarchical clustering of 30 the most abundant genera.

but was present also in eggshell waste and caeca of 4-day-old chicks. *E. coli* was the most frequent representative of family Enterobacteriaceae that was found in all sample types. In addition, *Klebsiella* was found in eggshell waste and caeca of 4-day-old chicks (Fig. 3) with *Klebsiella pneumoniae* and *Klebsiella variicola* as the likely species recorded.

Representatives of Clostridiaceae dominated in the caeca of one-day-old chicks. Three different species, *Clostridium paraputrificum, C. disporicum* and *C. tertium* were detected in gut microbiota of one-day-old chicks. Of these, *C. disporicum* was common in eggshell waste and *C. paraputrificum* could be also found in eggshell waste, though not as commonly as *C. disporicum* (Fig. 3).

Different species from family Lachnospiraceae were present in the caecum of one- and four-day-old chicks. *Zhenhengia yiwuensis* (*Epulopiscium*) was the only species from family Lachnospiraceae found in the caeca of one-day-old chicks. The remaining 55 ASVs of family Lachnospiraceae belonged to genera *Ruminococcus, Anaerostipes, Blautia, Lachnoclostridium, Sellimonas, Tuzzerella, Tyzzerella* or other unclassified Lachnospiraceae, and these were characteristic for caecal microbiota of 4-day old chicks (Fig. 3).

Representatives of family Bacteroidaceae were recorded in dead embryo samples and in the caeca of 4-day-old chicks. Species found in the former type of sample included *Bacteroides mediterraneensis, B. caecicola,*

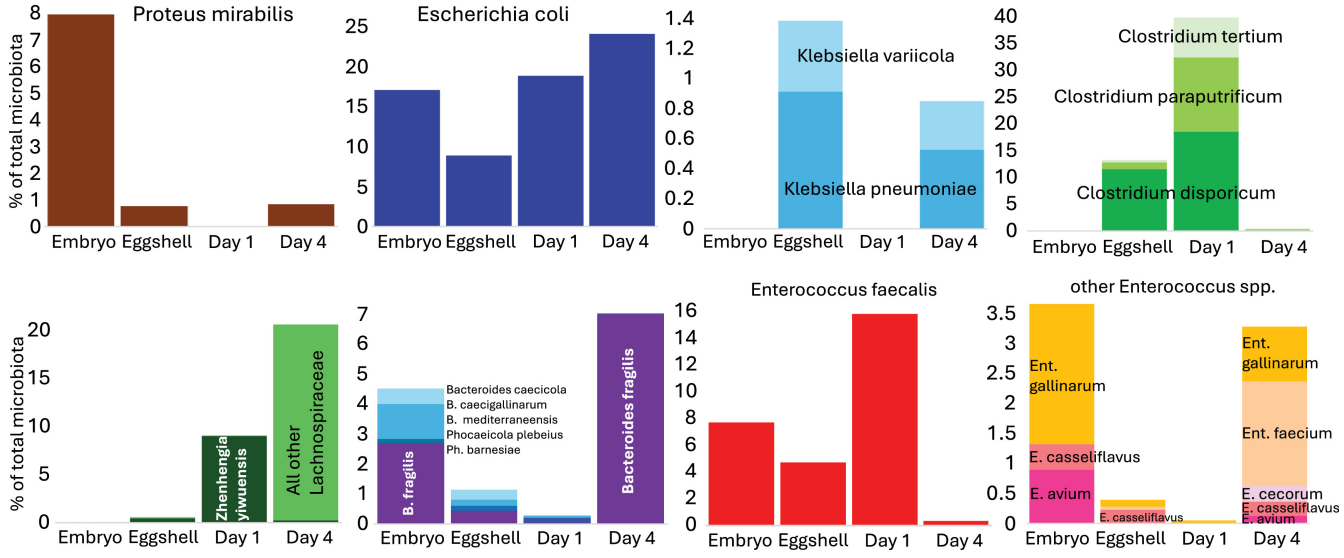


Fig. 3. Distribution of lower taxa across multiple sample types. Different ASVs identified down to species using ASV sequence by BLAST comparison to the GenBank entries exhibited different dominance across tested samples. Mind only Y axis scaling in individual panels indicating different dominance of shown taxa in individual samples.

B. caecigallinarum, Phocaeicola barnesiae and *P. plebeius*, i.e. chicken-adapted species, as well as *Bacteroides fragilis* which is usually associated with mammals (Kollarcikova et al., 2020). On the other hand, caeca of 4-day-old chicks were colonised by *B. fragilis* only (Fig. 3).

Six different species from genus *Enterococcus* were recorded across all sample types. *Ent. faecalis* was common in dead embryos, eggshell waste and caeca of one-day-old chicks but was virtually absent from 4-day-old chicks. *Ent. avium, Ent. gallinarum* and *Ent. casseliflavus* were common in dead embryo microbiota and in the caeca of 4-day-old chickens. *Ent. faecium* and *Ent. cecorum* were recorded in the caeca of 4-day-old chicks only and were nearly absent from other sample types (Fig. 3).

Qualitative distribution of E. coli and Ent. faecalis in caecal microbiota of one-day-old chicks

Microbiota of dead embryos and caeca of one-day-old chicks was commonly dominated by a single bacterial species resulting in low α diversity (Fig. 4A). While we refer to Supplementary File 1 for the dead embryo microbiota, the abundance of *E. coli* and *Ent. faecalis* in caecal microbiota of one-day-old chicks is shown in Fig. 4B and C. *Ent. faecalis* formed around 16 % of total caecal microbiota in one-day-old chicks on average. However, the use of average abundance is confounding in this case since the abundance of *Ent. faecalis* can be better viewed either as present or absent (Fig. 4B). The same was true also for *E. coli* with most of one-day-old chicks being either positive for *E. coli,* which then formed 50 to 95 % of total microbiota, or negative (Fig. 4C). Unlike *Ent. faecalis, E. coli* colonised the chicken caecum during the first days of life and the average abundance of *E. coli* in 4-day-old chickens was a representative value since all 4-day-old chicks were *E. coli* positive and *E. coli* abundance in the caeca of individual chicks fluctuated around an average 25 % abundance (Fig. 4C).

Abundance distribution of *E. coli* using Kernel Density Estimation confirmed the described pattern. *E. coli* distribution with 2 peaks was recorded in dead embryos and caecal samples from one-day-old chicks what showed that these samples were either negative or lowly positive for *E. coli*, or dominated by *E. coli*. In most eggshell waste samples, *E. coli* was abundant at a low level but there was a moderate probability that some of the eggshell waste samples could be dominantly populated by *E. coli* (Fig. 4D).

Culture confirmation of sequencing data

Since DNA sequencing or real-time PCR may detect DNA from non-viable bacteria, an additional 98 caecal samples from one-day-old chicks were subjected to aerobic cross-streak culture on MacConkey agar. Of these, *E. coli* growth was recorded in 15 samples. This means that the majority of chicks were negative for viable *E. coli* in the caecum on day 1 of life, in agreement with the sequencing data in Fig. 4C. Caecal contents were subjected also to culture on BHI, WCHA and blood agar under both aerobic and anaerobic conditions and *Clostridium tertium* was isolated after aerobic incubation, whereas *C. paraputrificum* and *C. disporicum* were detected after incubation under anaerobic conditions. Although we failed to culture *Terrisporobacter* and *Zhenhengia* (*Epulopiscium*), likely due to specific requirements of different gut anaerobes (Lagier et al., 2016; Crhanova et al., 2019), the viability was confirmed for at least all three *Clostridium* species.

Discussion

In this study, we compared the microbiota composition of dead chicken embryos, eggshell waste from hatcheries and the caeca of 1- and 4-day-old chicks. Multiple taxa characteristic of each sample type were identified, as well as those detected in similar abundances in more than one type of sample. Taxa with similar abundances in more than one sample types were proposed to be transmitted. While the likelihood of transmission increases with the higher taxon abundance in different samples, such conclusion cannot be understood as evidence. Taxa with low abundance in one type of sample and high abundance in another one may still be transferred since many bacteria can increase in counts within a few hours if shifted to favourable growth conditions.

Although number of samples collected from dead embryos was lower in comparison to other types of samples, microbiota in dead embryo sam-

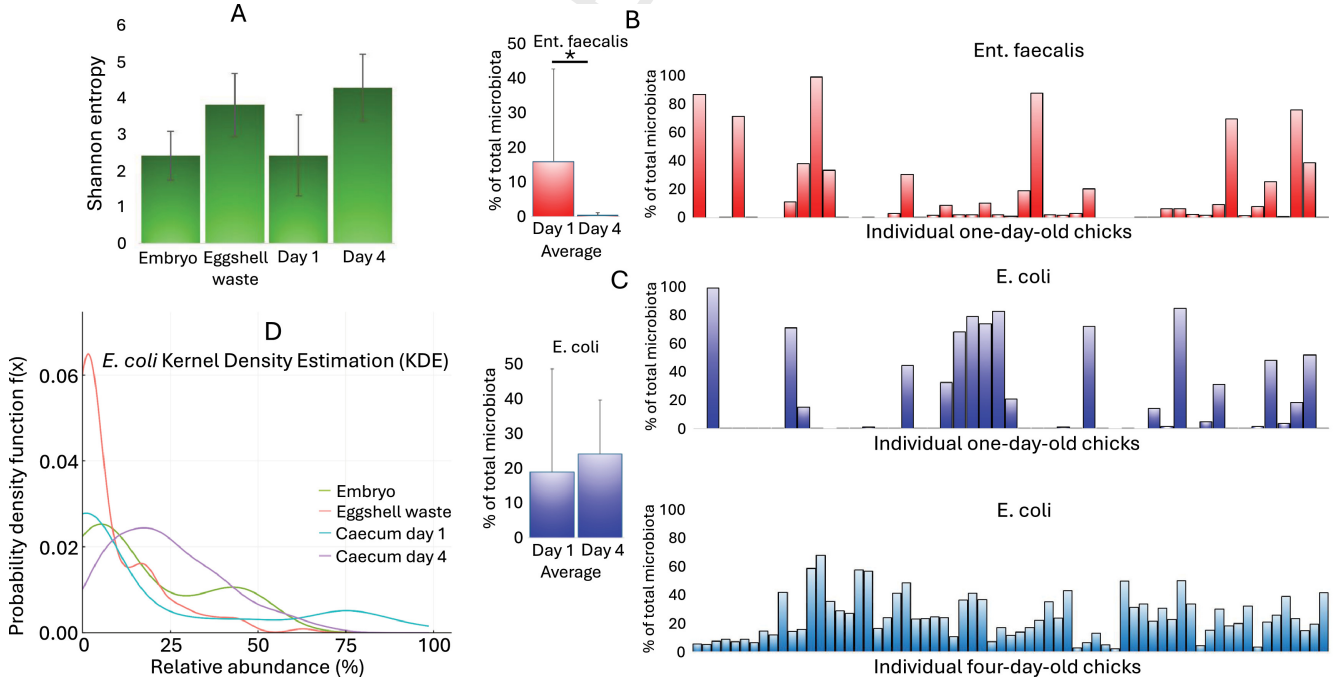


Fig. 4. Distribution of *E. coli* and *Ent. faecalis* in the caeca of one- and four-day-old chicks. Panel A, α diversity (Shannon entropy) in dead embryo, eggshell waste and caecal microbiota of one- and four-day-old chicks. Panel B, *Ent. faecalis* average abundance and abundance in the caeca of individual one-day-old chicks. Panel C, *E. coli* average abundance and abundance in the caeca of 49 individual one- and 67 four-day-old chicks. Panel D, *E. coli* abundance using Kernel Density Estimation. Data in panels A and D are derived from analysis of all 260 samples subjected to 16S rRNA sequencing. * - significantly different by t-test ($p < 0.05$).

ples was quite unique with the genera *Comamonas, Helcococcus, Brevibacterium* or *Proteiniphilum* being characteristic for this type of sample. *Comamonas testosteroni* as a cause of mortality in embryonated chicken eggs was reported earlier (Chaves-Hernandez et al., 2024). *Comamonas testosteroni* was recorded also in eggshell waste and dead embryos, though other *Comamonas* species dominated eggshell waste. The major dead embryo bacteria did not contaminate the eggs that underwent normal embryonic development as the eggshell waste microbiota was dominated by *Acinetobacter, Pseudomonas, Janthinobacterium, Stenotrophomonas, Chryseobacterium, Sphingobacterium, Empedobacter* or *Comamonas. Acinetobacter, Escherichia, Proteus* and *Pseudomonas* were detected on eggshells earlier (Trudeau et al., 2020) and *Klebsiella, Proteus, Pseudomonas, Sphingobacterium* and *Xanthomonas* were cultured from the eggshells in another study (Musgrove et al., 2008). Correlation analysis showed a negative interaction between *Chryseobacterium, Sphingobacterium, Empedobacter* or *Comamonas* at one side and *E. coli* or *Ent. faecalis* on the other side in eggshell waste, which can be used for the development of new competitive exclusion products.

Caecal microbiota of one-day-old chicks prior to any contact with feed and the farm environment was one of the most unexpected findings. *C. disporicum, C. paraputrificum, C. tertium, Terrisporobacter* and *Zhenhengia* (*Epulopiscium*) were commonly recorded even though these taxa are not usually reported as members of chicken caecal microbiota. Sequencing may detect also DNA from non-viable bacteria and this might be critical for samples with low bacterial biomass like caecum of newly hatched chicks. However, this can be excluded since we successfully grew 3 *Clostridium* species from this type of sample. In addition, the same bacteria were recorded in microbiota of newly hatched chicks earlier (Glendinning et al., 2019; Huang et al., 2023) showing that our findings were not affected by local conditions. *C. disporicum, C. paraputrificum* were also recorded on the skin of newly hatched chicks likely through contamination from faces in the external environment (Faldynova et al., 2024). Since these bacteria were present in the caecum before chicks began feed intake, these bacteria must forage on nutrients exclusively of chicken origin. In agreement, *C. disporicum* and *C. tertium* were reported to be able to use mucin proteins as a sole carbon source (Raimondi et al., 2021). Following the administration of feed rich in polysaccharides of plant origin, these bacteria were then either directly eliminated or eliminated indirectly via competition with gut microbiota specialising in carbohydrate fermentation (Polansky et al., 2015; Rajova et al., 2024). The caecal microbiota of four-day-old chicks was characterised by the presence of aerotolerant Lactobacilli and Streptococci, *E. coli* and spore-forming representatives of the families Lachnospiraceae, Oscillospiraceae and Ruminococcaceae. Such a developmental pattern has been described repeatedly (Stanley et al., 2013; Videnska et al., 2014; Xi et al., 2019) likely because of chicken inoculation by spores of these otherwise anaerobic bacteria surviving in the external environment (Rychlik, 2026).

Huang et al. reported the presence of *E. coli, Klebsiella, Enterobacter* and *Salmonella* in the caeca of one-day-old chicks (Huang et al., 2023). *Klebsiella, Enterobacter, Salmonella* and *Enterococcus* species different from *Ent. faecalis* were recorded in eggshell waste and then in the caecal microbiota of four-day-old chicks. However, these genera were usually absent from the caeca of one-day-old chicks. It is therefore impossible to decide whether these were transferred from hatcheries to chicks or whether these were newly acquired by chicks from the farm environment since negativity determined by DNA sequencing in the caecum of newly hatched chicks does not exclude a very low positivity. Alternatively, chicks leaving hatcheries can be colonised by these bacteria outside the caecum, e.g. on the skin or in the respiratory tract (Faldynova et al., 2024) from where they can colonise digestive tract.

For *Ent. faecalis* and *E. coli*, the transfer from hatcheries to chicks and farms was determined in greater detail. The abundance of *E. coli* and *Ent. faecalis* in the caecum of one-day-old chicks was qualitative with the majority of chicks being *E. coli* negative at the moment they were placed on farms. Nearly 85 % of chicks were free of *Ent. faecalis* and *E. coli* in the caecum, similar to previous reports (Laube et al., 2013; Projahn et al., 2017) what means that there is still an open window for interventions like probiotic administration when chicks are deployed at farms (Volf et al., 2024). The subsequent fate of *E. coli* and *Ent. faecalis* then differed. While *E. coli* appeared in all tested chicks 3 days later, *Ent. faecalis* decreased in the caecum between day 1 and day 4. This may indicate that the dissemination of *E. coli* and the decrease of *Ent. faecalis* and other early colonisers is dependent on feed intake. Longer transport of chicks to farms without access to feed therefore may increase the risk with *Ent. faecalis* inflammation of the egg yolk sac, while shorter transport and early access to feed may affect *E. coli* infections. *E. coli* dominance is, however, only temporary since from the second week of chicken life, representatives of Oscillospiraceae, Ruminococcaceae, Erysipelatoclostridiaceae, Butyricicoccaceae and Lachnospiraceae become dominant and outcompete the temporarily dominating *E. coli* (Stanley et al., 2013; Videnska et al., 2014; Xi et al., 2019).

Conclusions

We have shown that the major microbiota members of dead egg embryos, eggshell waste and caeca of one- and four-day-old are, in most cases, different and carry-over of bacteria from one sample type to another is uncommon. There are, however, several exceptions. *E. coli* and *Ent. faecalis* in one-day-old chicks likely arise from the hatchery environment and the same is likely for *Proteus, Klebsiella* and other *Enterococcus* species. The caeca of one-day-old chicks in hatcheries were colonised by *C. disporicum, C. paraputrificum, C. tertium, Terrisporobacter* and *Zhenhengia* (*Epulopiscium*). Whether these bacteria stimulate the chicken innate immune system in any way is currently unknown. Only 10-20 % of chicks leaving hatcheries were positive for *E. coli*. It would be interesting to test whether *E. coli* strains from chicks on day 4 of life represent new clones from the farm environment or the clones from hatcheries, which spread from chicks positive in hatcheries to the remaining chicks in the flock.

Supplementary information

Supplementary table 1. ASV table of all 16S rRNA sequences recorded at least once in this study.

Disclosures

The authors declare that they have no competing interests.

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Supplementary materials

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